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14. (new) A method for treating cancer, wherein the cancer cells selectively express the endothelin B receptor, comprising administering a compound that is an antagonist to an endothelin B receptor to a subject in need of such treatment.

15. (new) A method for treating cancer, comprising administering a compound that prevents the downregulation of E-cadherin in the cancer cell to a subject in need of such treatment.

REMARKS

Claims 1-5 are presently pending. Claims 1, 4, and 5 have been amended to more particularly point out and distinctly claim the invention; to place the claims in condition for allowance. The specification has been amended to include separate descriptions of Figures 4A-J in the Brief Description of the Drawings section. No new matter has been added (*see e.g.*, the instant specification on page 51, line 31 to page 52, line 19). New Claims 14 and 15 are fully supported by the instant specification. In particular, support can be found, e.g., on page 15, lines 22-24 and on page 48, lines 8-18. Support for the claim amendments may be found in the instant specification, thus, no new matter has been introduced. In particular, support for the amendment to Claim 1 can be found, *e.g.*, on page 15, lines 15-21; page 19, line 30 to page 20, line 18; and page 55, lines 3-7. Support for the amendment to Claim 4 can be found, e.g., on page 16, lines 1-6; page 22, lines 19-21; page 24, lines 8-11; and page 25, line 22-25. Support for the amendment to Claim 5 can be found, *e.g.* on page 17, lines 3-7. Entry of the foregoing amendments are respectfully requested.

A marked up version of the replacement paragraph and amended claim showing the amendments is attached hereto as Appendices A and B, respectively. Matter that has been deleted is indicated by brackets and matter that has been added is indicated by

underlining. A copy of the claims as pending after entry of the foregoing amendment is attached as Appendix C.

The Drawings

The drawings have been objected to and require correction. Submitted herewith as Appendix D are replacement informal Figures 1-4. Applicants will submit formal drawings should subject matter be found allowable.

The Rejections Under 35 U.S.C. § 112, First Paragraph Should Be Withdrawn

Claims 1-5 are rejected under 35 U.S.C. § 112, first paragraph, because the specification allegedly does not reasonably provide enablement commensurate with the scope of the claimed invention. The Examiner contends that the specification only teaches administration of endothelin receptor inhibitors to melanoma cells, as such, administration of any compound to any and all cancers other than melanomas is not enabled. This rejection is in error and should be withdrawn.

The specification teaches methods designed to target ET-1 mediated initiation of cancer (*see e.g.*, the instant specification on page 19, line 30 to page 20, line 2). The specification provides that the invention relates to the treatment and prevention of cancers including, but not limited to, melanoma, prostate cancer, colon cancer, ovarian cancer, and mammary cancers using compounds that antagonize the endothelin B receptor or result in the prevention of downregulation of E-cadherin (*see e.g.*, page 15 of the instant specification). The instant application has enabled the treatment of each of these cancers and, has provided evidence of such enablement through the data supplied in the Working Examples demonstrating the successful treatment of melanoma (see, the instant specification at pages 47-52). According to applicable case law, an inventor is not required to disclose "a test of every species encompassed by their claims" even in an unpredictable art. *In re Angstadt*, 190

U.S.P.Q. 214, 218 (C.C.P.A. 1976) (emphasis in original). Accordingly, the scope of the claims should not be limited to one species (*i.e.*, melanoma) when the methods of the present invention are applicable to a genus of cancers.

Cancers in the genus could be identified by one of ordinary skill in the art. A literature search of the NCBI PubMed database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed>) quickly revealed a number of post-filing publications describing cancers other than melanoma that could be treated by the addition of endothelin B receptor antagonists. For example, glioblastoma cancer cells were found to undergo apoptosis upon the addition of an endothelin receptor antagonist (Egidy et al., 2000, *Lab Invest* 80:1681-9; submitted herewith as Exhibit 1). Although the antagonist used was capable of inhibiting both types of endothelin receptors, the cancer cells were shown to express endothelin B receptor and ET-1 was mainly acting via that pathway. Additionally, breast cancer cells were shown to express elevated levels of endothelin B receptor as compared to their normal counterparts (Alanen et al., 2000, *Histopatholog* 36:161; submitted herewith as Exhibit 2). Even though cancers of differing cell types have been reported to respond differently to a given therapeutic approach, all cancers with a common basis (*e.g.*, ET-1 signaling through the endothelin B receptor) should all be improved by targeting that unifying characteristic (*e.g.*, decreased or inhibited signaling through the endothelin B receptor).

In view of the foregoing, the rejections under 35 U.S.C. § 112, first paragraph are obviated and should be withdrawn.

The Rejections Under 35 U.S.C. § 112, Second Paragraph Should Be Withdrawn

Claims 4 and 5 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. This rejection is in error and should be withdrawn.

The Examiner contends that the recitation of “the compound is a mimic” in claim 4 is vague and indefinite because it is not clear what aspect of endothelin-1 is to be mimicked. Applicants have amended claim 4 to recite the aspect of endothelin-1 that is of relevance – namely its ability to bind to the endothelin B receptor (see, *e.g.*, page 25, lines 22-25 of the instant specification).

The Examiner contends that the recitation of “molecule” in claim 5 is vague and indefinite because it is not clear what type of molecule that activates the endothelin B receptor is encompassed by the claim. Applicants have amended claim 5 to clarify that the type of molecule is a polypeptide (*i.e.*, the only type of molecule that is translated).

The Examiner contends that the recitation of “a molecule that activates endothelin B receptor” is vague and indefinite because the manner in which receptor activation occurs is unclear. Applicants assert that the *manner* of activation is not important – only that the receptor becomes active. Someone of skill in the art could use common molecular biological techniques to devise an *in vitro* assay to determine if the endothelin receptor has been activated without undue experimentation. The instant specification clearly discloses a number of consequences of receptor activation that could be used as a readout in such an assay including downregulation of E-cadherin, p120^{CTN}, and β -catenin as well as activation of caspase-8 (see, *e.g.*, page 15, lines 22-25 of the instant specification).

In view of the foregoing, the rejections under 35 U.S.C. § 112, second paragraph are obviated and should be withdrawn.

The Rejections Under 35 U.S.C. § 102 Should Be Withdrawn

Claims 1 and 4 are rejected under 35 U.S.C § 102(e) as being anticipated by U.S. Patent No. 5,382,569 (the “‘569 patent”). The Examiner contends that the ‘569 patent discloses novel antagonists of endothelin that are receptor antagonists useful in treating cancer. This rejection is in error and should be withdrawn.

The legal test for anticipation under 35 U.S.C. § 102 requires that each and every element of the claimed invention be disclosed in a prior art reference in a manner sufficient to enable one skilled in the art to reduce the invention to practice, thus placing the public in possession of the invention. W.L. Gore Associates v. Garlock, Inc., 721 F.2d 1540, 1554 (Fed.Cir. 1983) cert. denied 469 U.S. 851 (1984); In re Donohue, 766 F.2d 531 (Fed. Cir. 1985). Anticipation under 35 U.S.C. § 102 requires identity of invention. Scripps Clinic & Research Fdn. v. Genentech Inc., 927 F.2d 1565 (Fed. Cir. 1991).

In the present instance, the ‘569 patent does not describe a method of treating cancer comprising administering a selective antagonist for the endothelin B receptor; therefore, the invention is not anticipated.

The ‘569 patent describes that elevated levels of endothelin have been postulated to be involved in a number of diseases. The ‘569 patent provides a list of antagonists of endothelin which may be useful in treating disorders related to elevated levels of endothelin. Also disclosed are a laundry list of proposed disorders which may be candidates for treatment with the endothelin (ET-1) antagonists. Candidate disorders were selected due to reports in the literature of elevated levels of circulating ET-1 in the plasma of

individuals suffering from such disorders. Table I in column 5 of the '569 patent lists the diverse array of disorders compiled in this manner.

There is no identification or recognition in the '569 patent that a cancer can be treated by the administration of a compound that acts to selectively antagonize the endothelin B receptor. Further, there is no identification or recognition in the '569 patent that a cancer wherein the cancer cells selectively express the endothelin B receptor, can be treated by the administration of a compound that is an antagonist of the endothelin B receptor (new Claim 14). Furthermore, there is no identification or recognition in the '569 patent that a cancer can be treated by the administration of a compound that prevents the downregulation of E-cadherin (new Claim 15).

Thus, in view of the foregoing, as anticipation requires identity of the claimed invention, the '569 patent cannot anticipate the invention as claimed. Therefore, the rejection under § 102(e) in view of the '569 patent is in error and should be withdrawn.

In the present instance, Okazawa does not describe a method of treating cancer comprising administering a selective antagonist for the endothelin B receptor; therefore, the invention is not anticipated.

Claims 1-4 are rejected under 35 U.S.C § 102(a) as being anticipated by Okazawa et al., 1998 *J. Biol. Chem.* 273:12584-92 ("Okazawa"). The Examiner erroneously interprets Okazawa as disclosing a method of treating melanoma cancer comprising administering BQ788. This rejection is in error and should be withdrawn.

Okazawa reports experiments done to characterize the effects of ET-1, an antagonist of ET receptors, on a culture of cell cycle synchronized A375 melanoma cells. Such effects included the ability of ET-1 to induce cell death or apoptosis of the melanoma cells. Okazawa reports that an antagonist of ET receptors, such as BQ788, was able to

prevent ET-1 induced apoptosis of melanoma cells. BQ788 was used to rescue cell death of melanoma cells induced by ET-1. Thus, the Examiner is incorrect in his allegation that Okazawa teaches the use of BQ788 in the treatment of melanoma. Okazawa, in fact, teaches just the opposite. According to the teaching of Okazawa, one would administer an agonist of an ET receptor to induce cell death of a melanoma cell and administer an antagonist of an ET receptor to rescue the melanoma cell from cell death. Thus, there is no identification or recognition in Okazawa that a cancer can be treated by administering a compound that acts to selectively antagonize the endothelin B receptor. Further, there is no identification or recognition in Okazawa that a cancer that selectively expresses the endothelin B receptor, can be treated by the administration of a compound that is an antagonist of the endothelin B receptor (new Claim 14). Furthermore, there is no identification or recognition in Okazawa that a cancer can be treated by the administration of a compound that prevents the downregulation of E-cadherin (new Claim 15).

Thus, in view of the foregoing, as anticipation requires identity of the claimed invention, Okazawa cannot anticipate the invention as claimed. Therefore, the rejection under § 102(a) in view of Okazawa is in error and should be withdrawn.

In view of the foregoing, the rejections under 35 U.S.C. § 102 should be withdrawn.

MISCELLANEOUS

Claim 5 was objected to because of the inclusion of the term “or ribozyme”. The Examiner contends that ribozymes are the subject matter of Group II rather than elected Group I in the Restriction Requirement mailed November 17, 2000 in connection with the instant application. Consequently, Applicants have deleted the term objected to from claim 5

without prejudice, but reserve the right to prosecute the deleted subject matter in future continuation, continuation-in-part, or divisional applications.

CONCLUSION

Applicants respectfully request entry and consideration of the foregoing amendments and remarks. The claims are believed to be free of the art, and patentable.

Withdrawal of all the rejections and objections and allowance is earnestly sought.

Date August 23, 2001

Respectfully submitted,

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Enclosure



APPENDIX A

Marked-Up Copy of the Amended Paragraph
U.S. Patent Application Serial No. 09/305,084
Attorney Docket No. 5914-080-999

Figure 4: ET-1 alters the subcellular localization of E-cadherin and β -catenin.

Cells were incubated either with or without [stimulated for 96 hours with] 10nM ET-1 for 96 hours then fixed and stained with [either] anti- E-cadherin or anti- β -catenin antibodies followed by anti-mouse-IgG-Cy3 antibodies. E-cadherin localization is shown for melanocytes either (A) without or (B) with ET-1 incubation and in melanoma cells either (C) without or (D) with ET-1 incubation. β -catenin localization is shown for melanoma cells either (E) without or (F) with ET-1 incubation and in melanocytes either (G) without or (H) with ET-1 incubation. Melanocyte cell morphology is shown by bright field micrographs of cells either (I) without or (J) with ET-1 incubation. Melanocyte cell morphology is shown by bright-field micrographs of cells either (I) with or (J) without ET-1 incubation. Incubation of melanocytes and melanoma cells with secondary antibody alone revealed no background staining.



APPENDIX B

Marked-Up Copy of the Amended Claims
U.S. Patent Application Serial No. 09/305,084
Attorney Docket No. 5914-080-999

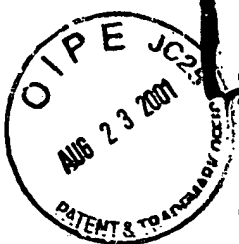
1. A method for treating a cancer, comprising administering a compound that is [an] a selective antagonist to an endothelin B receptor [(ETB)] to a subject in need of such treatment.
4. The method of Claim 1, in which the compound is a mimic of Endothelin-1 that binds to the endothelin B receptor.
5. The method of Claim 1 in which the compound is an antisense [or ribozyme] molecule that blocks translation of a polypeptide [molecule] that activates the endothelin B receptor [ETB].

APPENDIX C

Pending Claims as of August 23, 2001
U.S. Patent Application Serial No. 09/305,084
Attorney Docket No. 5914-080-999

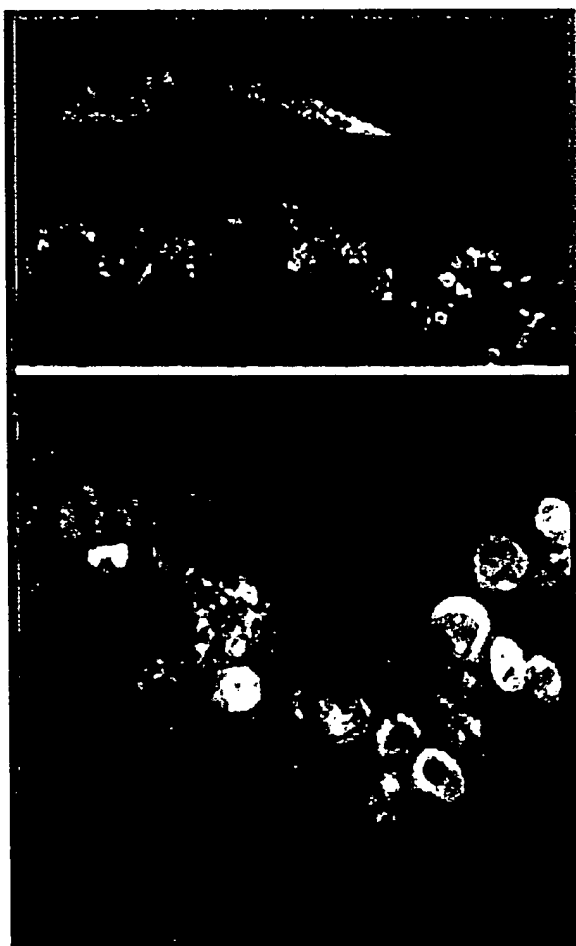
1. (amended) A method for treating a cancer, comprising administering a compound that is a selective antagonist to an endothelin B receptor.
2. The method of Claim 1 in which the cancer is selected from the group consisting of melanoma, prostate cancer, colon cancer, ovarian cancer or mammary cancer.
3. The method of Claim 2 in which the cancer is melanoma.
4. (amended) The method of Claim 1, in which the compound is a mimic of Endothelin-1 that binds to the endothelin B receptor.
5. (amended) The method of Claim 1 in which the compound is an antisense molecule that blocks translation of a polypeptide that activates the endothelin B receptor.
14. (new) A method for treating cancer, wherein the cancer cells selectively express the endothelin B receptor, comprising administering a compound that is an antagonist to an endothelin B receptor to a subject in need of such treatment.

15 (new) A method for treating cancer, comprising administering a compound that prevents the downregulation of E-cadherin in the cancer cell to a subject in need of such treatment.

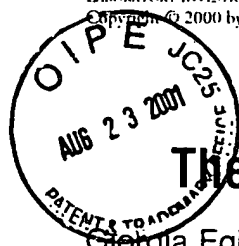


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The Endothelin System in Human Glioblastoma

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SUMMARY: Endothelin-1 (ET-1) is a powerful mitogenic and/or anti-apoptotic peptide produced by many cancer cells. To evaluate the potential role of the endothelin system in glioblastoma we first determined the cellular distribution of the mRNA and proteins of the components of the endothelin system, preproendothelin-1 (PPET-1), endothelin-converting enzyme-1 (ECE-1), and ET_A and ET_B receptors in human glioblastoma tissue and glioblastoma cell lines. PPET-1, ECE-1, and ET_A receptor were highly expressed in glioblastoma vessels and in some scattered glioblastoma areas whereas ET_B receptor was mainly found in cancer cells. This suggests that glioblastoma vessels constitute an important source of ET-1 that acts on cancer cells via the ET_B receptor. Four human glioblastoma cell lines expressed mRNA for all of the components of the ET-1 pathway. Bosentan, a mixed ET_A and ET_B receptor antagonist, induced apoptosis in these cell lines in a dose-dependent manner. Apoptosis was potentiated by Fas Ligand (APO-1L, CD95L), a pro-apoptotic peptide, only in LN2308 cells, corresponding to the known functional Fas expression in these cell lines. LN2308 cells also expressed the long and short forms of the cellular FLICE/caspase-8 inhibitory protein (FLIP). Bosentan and a protein kinase C inhibitor down-regulated short FLIP in these cells. ET-1 induced transient phosphorylation of extracellular signal-regulated kinase but did not induce long-term thymidine incorporation in LN2308 glioblastoma cells. These results suggest that, in glioblastoma cells, ET-1, mainly acting via the ET_B receptor, is a survival/anti-apoptotic factor produced by tumor vasculature, but not a proliferation factor, involving protein kinase C and extracellular signal-regulated kinase pathways, and stabilization of the short form of FLIP. (*Lab Invest* 2000, 80:1681-1689).

Endothelins (ET) (Yanagisawa et al, 1988) 1, 2, and 3 are a family of 21 amino-acid peptides enzymatically released from 200-residue prepolypeptides (Inoue et al, 1989) by furin-like activity to produce first inactive big ET (Denault et al, 1995). They are then cleaved by endothelin-converting enzyme (ECE-1) to yield the active peptides (Shimada et al, 1995). At least 4 isoforms, ECE-1_{a-d} have been characterized (Valdenaire et al, 1999). ET act on two distinct high-affinity receptor subtypes, ET_A (Arai et al, 1990) and ET_B (Sakurai et al, 1990), which belong to the seven transmembrane G-protein-coupled receptor family. At physiologic concentrations, ET-1 and ET-2, but not ET-3, bind to ET_A receptors, whereas all three ET ligands bind ET_B receptors with a similar affinity. In addition to its vasoconstrictor activity, ET-1 is a potent autocrine/paracrine mitogen in many cell types, including neoplastic cells and astrocytes, involving the

extracellular signal-regulated kinase (ERK) intracellular signaling pathway (Kusuhara et al, 1990; Shichiri et al, 1991; Teixeira et al, 2000). The expression of endothelins and endothelin receptors at the protein level has been shown in human glioma (Harland et al, 1995, 1998; Stiles et al, 1997; Tsutsumi et al, 1994).

Recently it has been suggested that ET-1, in addition to its mitogenic effects, may also regulate apoptosis in rat endothelial cells (Shichiri et al, 1997) and human smooth muscle cells (Wu-Wong et al, 1997). Apoptosis is an active cell death process that takes place in a wide spectrum of physiologic situations such as normal cell turnover, embryogenesis, and endocrine-dependent tissue atrophy. Interaction between the Fas receptor (CD95/APO-1), a member of the TNF-receptor superfamily, and the Fas ligand (FasL), triggers a pathway to cell death involving caspase activity. Although the expression of Fas and FasL has been demonstrated in glioblastoma (Saas et al, 1997), not all glioblastoma cell lines were able to respond to Fas engagement (Gratas et al, 1997). Tumor cell resistance to FasL-induced apoptosis has been described in different cancers by various mechanisms, including upregulation of caspase inhibitory molecules such as the pro-caspase-8 (FLICE)/caspase-8 inhibitory proteins (FLIP) (Irmeler et al, 1997). We have recently shown that ET-receptor blockade by bosentan, a mixed ET_A/ET_B receptor antagonist, and two receptor subtype-specific antagonists, BQ123 (ET_A selective) and BQ788 (ET_B selective) sensitized

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Fas-expressing colon carcinoma cells, which were resistant to FasL-induced apoptosis, to FasL-mediated apoptosis (Peduto Eberl et al, 2000, 2000 in press).

These previous studies indicated a potential function for ET-1 in cell proliferation or as a cell survival factor. To evaluate the role of ET-1 in glioblastoma, we determined the expression of the complete ET-1 system in human glioblastoma and glioblastoma cell lines, then evaluated its role in human glioblastoma cell survival and proliferation.

Results

Endothelin System mRNA and Proteins in Human Glioblastoma

All of the components of the endothelin system were highly expressed in glioblastoma. In situ hybridization (ISH) localized preproendothelin-1 (PPET-1) and ECE-1 mRNA expression mainly to the tumor vascu-

lature (Fig. 1a to c). ECE-1 mRNA signal colocalized with *Ulex europaeus* immunoreactivity (Fig. 1e) in endothelial cells and with ECE-1 immunoreactivity in tumor vasculature and in some tumor cells (Fig. 1f). PPET-1 mRNA was detected almost exclusively in endothelial cells (Fig. 1d), whereas ET-1 immunoreactivity was observed in α -smooth muscle actin- (α -SMA) immunoreactive cells in the vasculature, and in cancer cells (Fig. 1g), but not in all vessels (Fig. 1h). A perinuclear pattern of ET-1 immunoreactivity was evident in some glioblastoma areas (Fig. 1i).

ET-1 receptor distribution was evaluated using ISH (Fig. 2a to h) and [125 I]-ET-1 binding autoradiography (Fig. 2i to m). ET_A receptor mRNA was expressed in the tumor vasculature (Fig. 2a to d) and colocalized with CD31-immunoreactive endothelial cells (Fig. 2b), in microvessels (Fig. 2d), and in the glomeruloid α -SMA-immunoreactive vascular structures observed in glioblastoma and in few scattered tumor cells (Fig. 2c). ET_B receptor mRNA was almost exclusively ob-

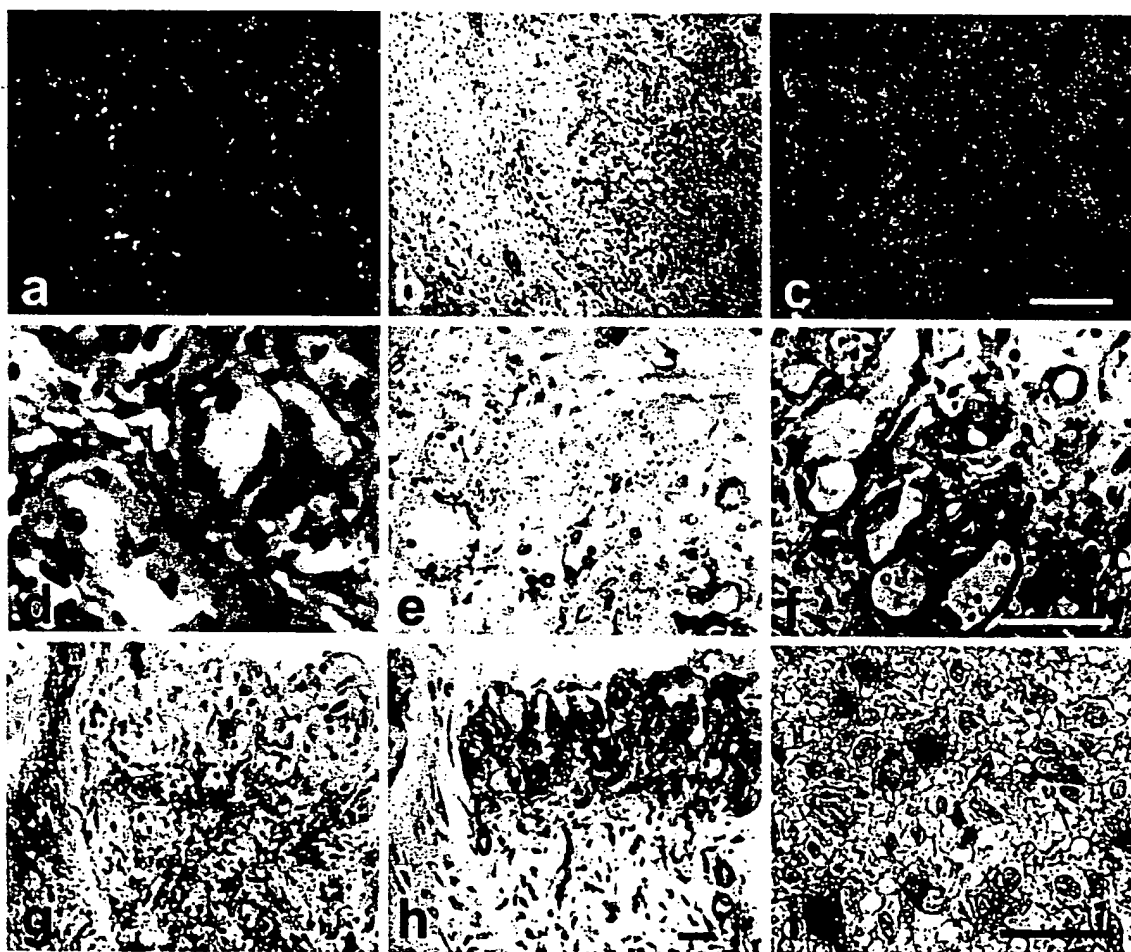


Figure 1.

Endothelin-1 (ET-1) and endothelin converting enzyme-1 (ECE-1) expression in human glioblastoma. In situ hybridization (ISH) was performed with the antisense probe for preproendothelin-1 (PPET-1) (a, b, d) and ECE-1 (c, e); and histochemistry with *Ulex europaeus* (e), ECE-1 (f), ET-1 (g, i), and α -smooth muscle actin (α -SMA) (h) antibodies. Darkfield (a, c) and bright-field illumination (b) of the same glioblastoma area in consecutive sections showed vascular expression of PPET-1 and ECE-1 mRNA. At higher magnification, PPET-1 (d) and ECE-1 (e) mRNA and ECE-1 immunoreactivity (f) were mainly expressed in endothelial cells as shown by colocalization of ECE-1 with *Ulex europaeus* (e). ET-1 immunoreactivity was found in α -SMA-immunoreactive cells, but not in glomeruloid structures (h) and in a perinuclear location in some glioblastoma cells (i). Scale bar = 100 μ m in a to c, 20 μ m elsewhere. Sense probes yielded no specific labeling and immunohistochemistry without primary antibody did not result in nonspecific reactivity (not shown).

served in glioblastoma cells (Fig. 2e to g), and restricted to a few scattered endothelial cells (Fig. 2h). To assess the presence of functional ET_A and ET_B receptors in human glioblastoma, we performed [^{125}I]-ET-1 binding in frozen samples (Fig. 2i to m). [^{125}I]-ET-1 (Fig. 2i) bound specifically to glioblastoma cells and vessels. Nonspecific binding that remained after displacement with ET-1 was uniformly low (Fig. 2j). Identification of receptor subtype was performed in consecutive sections by competition with the selective ET_B receptor agonist sarafotoxin 6c, which showed the presence of the ET_A receptor (Fig. 2k), and also with the selective ET_A receptor antagonist, BQ123, which indicated the presence of the ET_B receptor (Fig. 2l). The presence of ET_A receptor was observed in tumor vasculature and the ET_B receptor was found in glioblastoma cells, confirming the ISH results. This

pattern of distribution was very similar in all tumor specimens. Table 1 summarizes the distribution of the endothelin system in human glioblastoma.

Role of ET-1 in Human Glioblastoma

RT-PCR analysis of four human glioblastoma cell lines, LN18, LN215, LN2308, and LN319, demonstrated the presence of all of the components of the endothelin system, including the ECE-1 $_{a-d}$ isoforms, PPET-1, PPET-3, and ET_A and ET_B receptors (Fig. 3). These cells secreted low levels of ET-1 in the presence of FCS over 48 hours (LN18: 8.9 pg/ml, LN215: 1.7 pg/ml, LN2308: 2.8 pg/ml, and LN319: <0.3 pg/ml). ET-1 secretion was below detection limits in the absence of FCS. These cells expressed low levels of

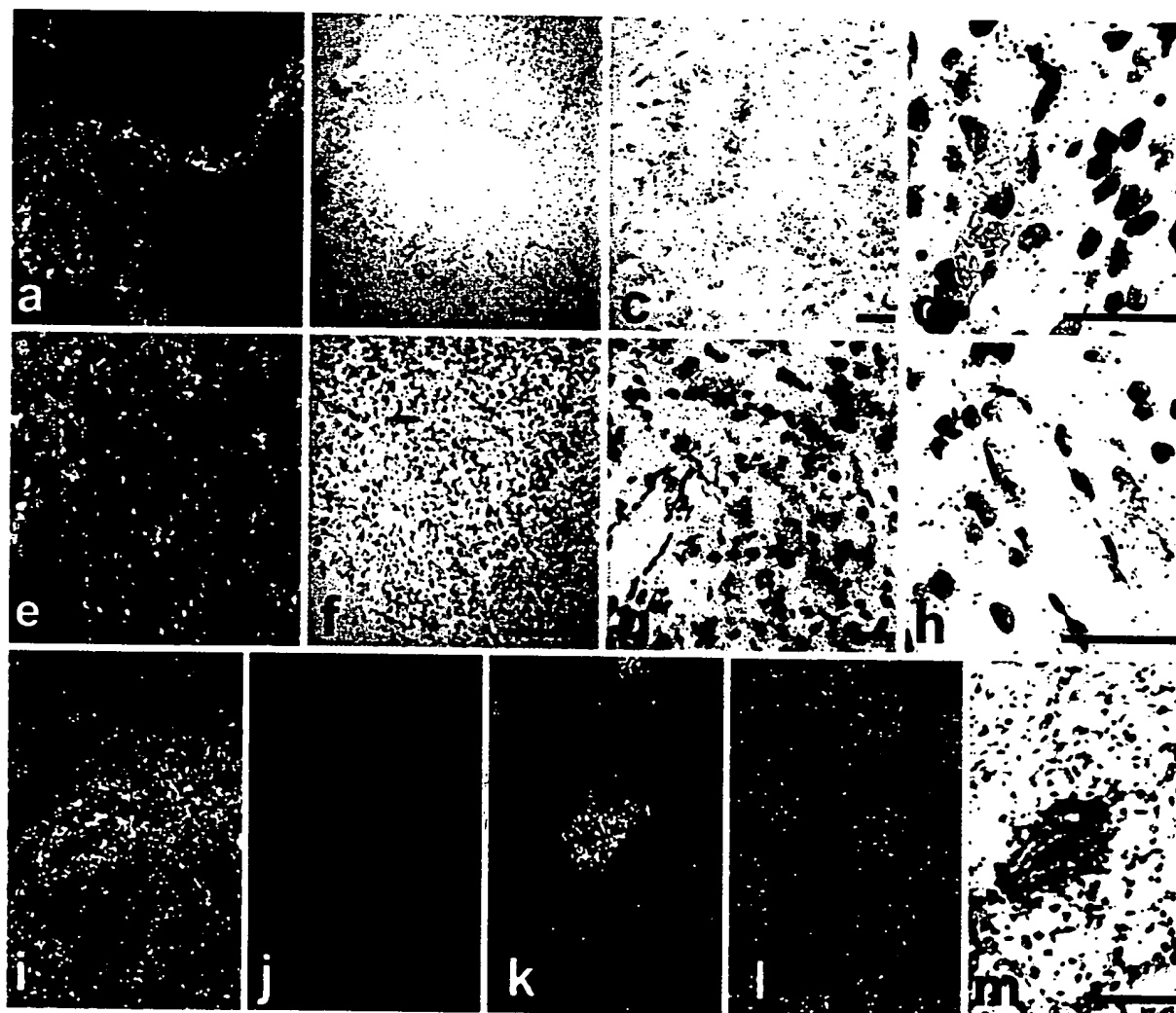


Figure 2.

ET_A and ET_B receptors in human glioblastoma. Darkfield (a, d) and bright-field illumination (c, e, f, h) of ISH performed with the anti-sense probes for ET_A receptors (a to d) and ET_B receptors (e to h) in human glioblastoma. Autoradiographic [^{125}I]-ET-1 (100 pM) binding was performed in frozen consecutive sections (i to m). Whereas the ET_A receptor showed a vascular distribution pattern demonstrated by colocalization with CD31 (b) immunoreactivity, the ET_B receptor (e) displayed a homogeneous distribution pattern in cancer cells. At high magnification, ET_A receptor mRNA was expressed in the tumor vasculature (c, d) and ET_B receptor in tumor cells (g), and some endothelial cells (h). ET-1 binding was used to ascertain functional receptor expression. i, Total ET-1 binding; j, nonspecific ET-1 binding; k, ET_A receptor binding in the presence of the ET_B receptor antagonist sarafotoxin 6c; l, ET_B receptor binding in the presence of the ET_A receptor antagonist BQ123; m, bright-field illumination of the same glioblastoma area. Scale bar = 100 μ m in a, b, e, f, i to m; 20 μ m in c, d, g, h. Sense probes yielded no specific labeling and immunohistochemistry without primary antibody did not result in nonspecific reactivity (not shown).

Table 1. Expression of the Endothelin System in Human Glioblastoma Vessel and Tumor^a

	mRNA		Protein	
	Glioblastoma cells	Vessels	Glioblastoma cells	Vessels
PPET-1/ET-1	—	+++	+++	++
ECE-1	+, s	+++	++	+++
ET _A	—	+++	—	+++
ET _B	+++	+, s	+++	+, s

ET-1, endothelin-1; PPET-1, prepro ET-1; ET_A, endothelin A receptor; ET_B, endothelin B receptor; s, scattered; +++, high expression; ++, medium expression; +, low expression; —, no expression observed.

^a mRNA expression was evaluated by in situ hybridization, PPET-1 and ECE-1 proteins by immunohistochemistry and ET receptors from binding autoradiography.

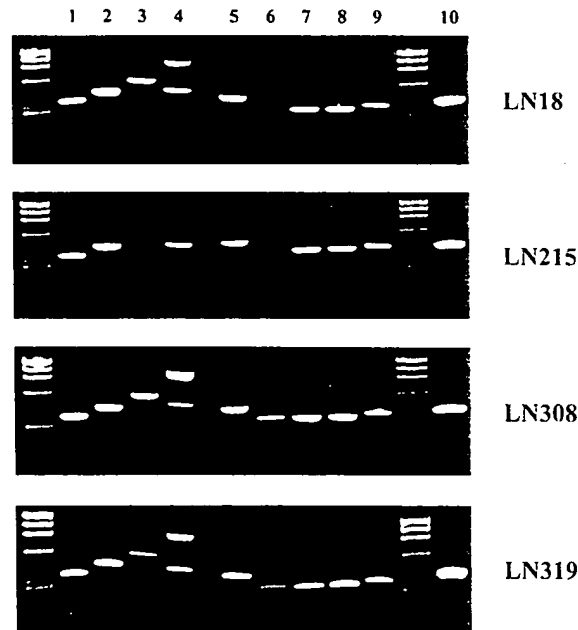


Figure 3.

Components of the endothelin system in human glioblastoma cell lines. RNA from four glioblastoma cell lines, LN18, LN215, LN308, and LN319, were used for RT-PCR analysis for PPET-1, PPET-3, ECE-1, ET_A and ET_B receptors, and GADPH, using the specific primers described in Table 2. Twenty-eight cycles of amplification gave each transcript at the expected size. Lane 1: 354 bp (PPET-1); lane 2: 445 bp (PPET-3); lane 3: 576 bp (ET_A receptor); lane 4: 477 bp (ET_B receptor); lane 5: 459 bp (ECE-1); lane 6: 353 bp (ECE-1a); lane 7: 347 bp (ECE-1b); lane 8: 348 bp (ECE-1c); lane 9: 369 bp (ECE-1d); and lane 10: 403 bp (GADPH). Molecular weight markers are Φ 174DNA/HaeIII. The high molecular weight band in the ET_B receptor lane is genomic DNA amplification.

ECE-1 as assessed by immunohistochemistry and western blotting (data not shown).

Increasing concentrations of bosentan, a mixed ET_A/ET_B receptor antagonist, induced apoptosis in the four glioblastoma cell lines in a dose-dependent manner (Fig. 4, open symbols). Addition of FasL-containing supernatants increased apoptosis only in LN308 cells. LN18 cells were sensitive to FasL-containing supernatants in the absence of bosentan, whereas addition of FasL-containing supernatants to LN215 and LN319 glioblastoma cells did not potentiate bosentan-induced apoptosis (Fig. 4, closed symbols). The addition of Neuro2A-control medium alone or in the presence of bosentan had no effect in any conditions (not shown). Rat glioblastoma Fas-positive F98

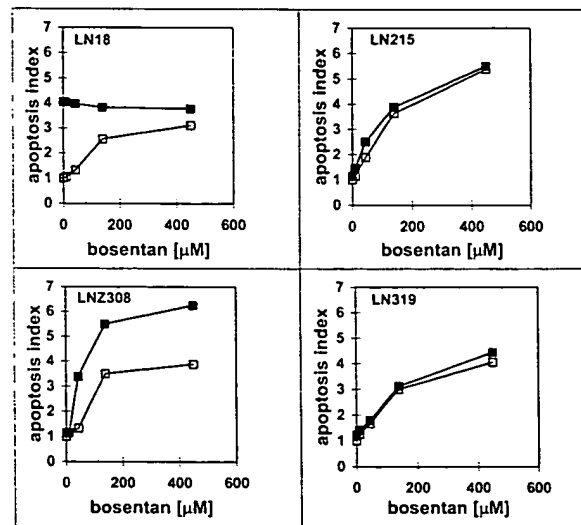


Figure 4.

Effect of bosentan and FasL on glioblastoma cell apoptosis. Fifty percent-confluent glioblastoma cells were incubated with increasing concentrations of bosentan (10 to 450 μ M) in the absence (\square) or in the presence (\blacksquare) of FasL-containing supernatant from Neuro2A cells. Apoptosis was evaluated after 24 hours of incubation. One representative experiment out of three is shown.

and Fas-negative F98ZH (Ambar et al, 1999) cells exposed to FasL-containing supernatants in the presence of bosentan were used as controls for the Fas/FasL dependency of the system (not shown).

Fas/FasL-responsive bosentan-sensitive LN308 cells were used to evaluate the roles of ET-1 and bosentan in glioblastoma apoptosis. Addition of 40 nM ET-1 (not shown), 0.4 nM ET-1 (Fig. 5a) or 4 pM ET-1 (not shown) induced rapid and transient p44/42 mitogen-associated protein (MAP) kinase/ERK phosphorylation in LN308 cells, demonstrating the presence of functional receptors and intracellular pathways in these cells. Addition of increasing concentrations of ET-1 for 3 hours, 5 hours, or 48 hours to glioblastoma cells, after 24 hours of FCS deprivation, did not increase thymidine incorporation (Fig. 5b), demonstrating that, at least in these glioblastoma cell lines, ET-1 is not importantly involved in sustained glioblastoma cell proliferation. Identical information was obtained using LN18, LN215, and LN319 cells (not shown).

Activation of the Fas death pathway involves the activation of FLICE, which can be antagonized by

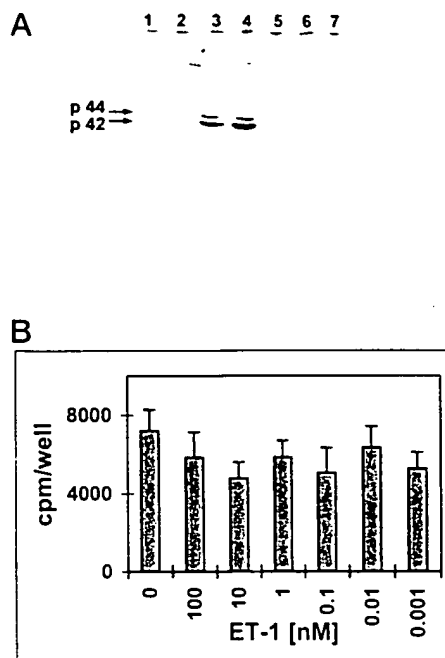


Figure 5.

Response of glioblastoma cells to exogenous ET-1. a: Extracellular signal-regulated kinase (ERK) phosphorylation. After 24 hours of FCS deprivation, LN2308 cells were incubated with 0.4 nM ET-1. Lane 1: no ET-1; lane 2: FCS, 120 minutes; lane 3: ET-1, 5 minutes; lane 4: ET-1, 10 minutes; lane 5: ET-1, 30 minutes; lane 6: ET-1, 60 minutes; lane 7: ET-1, 120 minutes. b: DNA synthesis. After 24 hours of FCS deprivation, LN2308 cells were incubated simultaneously with increasing concentrations of ET-1 and [3 H]-thymidine for 48 hours. Values are the means \pm SEM of two independent determinations performed in triplicate wells.

cellular inhibitors of caspase activation. Cellular FLIP is an inhibitory molecule potentially involved in resistance to FasL-induced apoptosis. Two forms of FLIP, a long and a short form, have been described (Irmeler et al, 1997). To evaluate the possibility that bosentan sensitization to FasL-mediated apoptosis in LN2308 cells may involve FLIP down-regulation or inactivation, we analyzed the expression of FLIP in glioblastoma cells. Glioblastoma cell lines expressed the short and long forms of cellular FLIP, as assessed by RT-PCR (Fig. 6) and expressed caspase-8, as assessed by western blotting (not shown). The short and long forms of FLIP mRNA were still expressed (not shown) in bosentan-treated cells 24 hours after exposure. However, in LN2308 cells exposed to high concentrations of bosentan alone or in combination with FasL-containing supernatant, the concentration of the short form, but not the long form, of the FLIP protein was decreased (Fig. 7a). The highest concentration of bosentan reproduced the effect of 10 μ M of the protein kinase C (PKC) inhibitor, bisindolylmaleimide IX (Fig. 7b).

Discussion

ET-1 was previously shown to be produced by human cancer cell lines and may be a growth promoting and survival factor for tumor and stromal cells. The expression of ET-1, ET-3, and their receptors has been

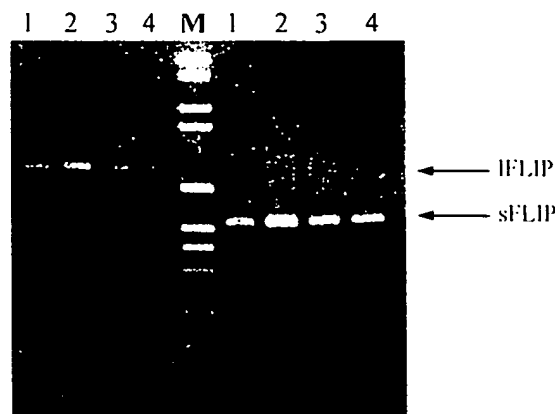


Figure 6.

Expression of long and short forms of cellular pro-caspase-8 (FLICE)/caspase-8 inhibitory protein (FLIP) by RT-PCR in four human glioblastoma cell lines. RT-PCR analysis for short and long FLIP was performed on total RNA extracted from the four glioblastoma cell lines using the specific primers described in Table 2. M: DNA marker. Two independent experiments gave similar results. Lane 1: LN18 cells; lane 2: LN215 cells; lane 3: LN2308 cells; lane 4: LN319 cells. Molecular weight markers are Φ 174DNA/HaeIII.

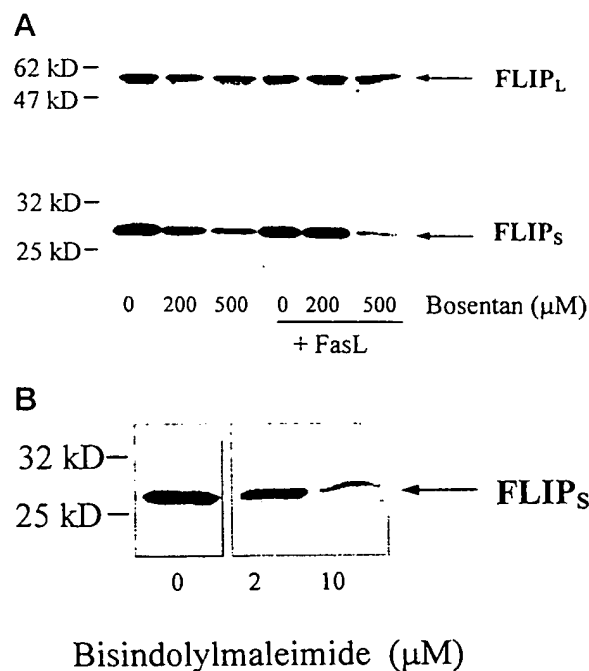


Figure 7.

Expression and regulation of caspase-8 and of long and short forms of cellular FLIP in the human glioblastoma cell line LN2308. Expression of caspase-8 and of the long and short forms of FLIP by bosentan and FasL (a) or the protein kinase C (PKC) inhibitor bisindolylmaleimide IX (b) were analyzed by Western blotting. a: Short and long FLIP in LN308 cells exposed to bosentan and FasL for 24 hours. b: Short FLIP in LN308 cells exposed for 24 hours to the PKC inhibitor bisindolylmaleimide IX. Two independent experiments gave similar results.

demonstrated in the brain, and in human glioblastoma (Harland et al, 1995, 1998; Tsutsumi et al, 1994). Glioblastoma also express the death-inducing receptor Fas (Saas et al, 1997). However, not all human glioblastoma cell lines are sensitive to Fas-induced death while expressing Fas (Gratas et al, 1997). Re-

cently, we have shown that colon cancer overexpresses all of the components of the ET-1 system (Egidy et al, in press) and that ET-1 is involved in the resistance of colon tumor cell lines to Fas-induced death (Peduto Eberl et al, 2000, 2000 in press). We hypothesized that ET-1 may also be involved in the proliferation or survival of glioblastoma, either through an autocrine effect on tumor cells or a paracrine effect on cancer-associated stromal cells. To evaluate this hypothesis, we examined the expression and potential function of the endothelin system components, including PPET-1, ECE-1, and ET_A and ET_B receptors in human glioblastoma and human glioblastoma cell lines.

To our knowledge, no studies have been reported on the precise cellular distribution of ECE-1 in relation to the other components of the ET-1 pathway, PPET-1, ET_A and ET_B receptors, in human glioblastoma. Our results demonstrated that mRNA for the substrate, PPET-1, its converting enzyme, ECE-1, and ET_A receptors were mainly expressed in tumor vessels, whereas ET_B receptors were almost exclusively expressed by tumor cells. The expression of ECE-1 protein was mainly vascular, although it could be detected in some locations in the tumors. A higher proportion of cells was detected with the ET-1 antibody than with the PPET-1 probes. This suggests immunoreactivity of receptor-bound ET-1, either at the cell membrane or to internalized receptors, and a paracrine role for vascular-derived ET-1. The distribution of ET_A and ET_B receptors determined by ET-1 binding corresponded to the distribution observed using ISH. ET_A receptors were highly expressed in vascular structures, α -SMA-immunoreactive cells, and endothelial cells, which confirms previously reported results (Harland et al, 1995, 1998; Tsutsumi et al, 1994), and in the glomeruloid structures of glioblastoma vasculature. ET_B receptors were found mainly in glioblastoma cells, and in only a very few endothelial cells. Thus, major differences are observed in cellular distribution, and consequently, the functions, of the ET-1 system between glioblastoma and colon carcinoma, in which ET_A and ET_B receptors were mainly observed in tumor-associated vasculature, myofibroblasts, and endothelial cells, and PPET-1 and ECE-1 were expressed in cancer cells (Egidy et al, in press).

What is the role of the endothelin system in glioblastoma? We used human glioblastoma cell lines to address this question. All components of the ET-1 system were detected by RT-PCR in four human glioblastoma cell lines. These cells secreted low levels of ET-1, corresponding to the low expression of PPET-1 observed by ISH in glioblastoma. They responded to ET-1 by transiently phosphorylating ERK, demonstrating the presence of functional ET-1 receptors and intracellular signaling pathways in the glioblastoma cell lines. ERK phosphorylation in response to ET-1 has been previously demonstrated in primary rat astrocytes (Sasaki et al, 1998; Teixeira et al, 2000), thus, astrocyte-derived human tumor cells have maintained this response to ET-1. However, ET-1 is not an important growth factor for glioblastoma cells for

long-term proliferation. The ability of ET-1 to stimulate ERK phosphorylation was mediated by PKC activation in airway smooth muscle cells (Whelchel et al, 1997), but not in Rat-1 cells (Cadwallader et al, 1997).

Bosentan (Clozel et al, 1993), a mixed ET_A/ET_B receptor antagonist, induced apoptosis in all glioblastoma cell lines in a dose-dependent manner, but potentiated FasL-mediated apoptosis only in the LN2308 cells, at concentrations below those needed to induce apoptosis with bosentan alone. In LN18 cells, FasL induced death in the absence of bosentan, whereas in LN215 and LN319 cells, FasL had no effect. It was previously shown that the cell lines used in this study express Fas and FasL, and that Fas engagement induced apoptosis only in LN18 cells (Gratas et al, 1997). Our results confirm those findings. In rat glioblastoma F98 cells, which express functional Fas, bosentan potentiated the FasL effect, whereas in Fas-negative F98ZH cells (Ambar et al, 1999), no potentiation of the effect was observed, demonstrating the Fas/FasL dependency of bosentan effects in glioblastoma.

The death receptor Fas is a member of the tumor necrosis factor family. Upon interaction with its ligand, it activates caspase-8 and induces cell death. However, despite expression of Fas and caspase, death signals are frequently interrupted by anti-apoptotic modulators, including caspase inhibitory proteins such as FLIP (Irmeler et al, 1997). Glioblastoma cell lines expressed caspase-8 protein and the long and short forms of FLIP, assessed by RT-PCR. Treatment of LN2308 cells with bosentan alone or in combination with FasL resulted in a decreased level of only the short form of FLIP protein, whereas short FLIP mRNA was not suppressed in these cells. This suggests that either translation is diminished or short FLIP degradation is increased. A similar effect on short FLIP was observed after treatment with a PKC inhibitor, suggesting that the bosentan effect is mediated by inhibition of PKC function. However, the high concentrations of bosentan necessary to obtain this effect indicate that the bosentan action is not likely to be mediated by cell membrane ET-1 receptors, but may involve intracellular components. In rat (Peduto Eberl et al, 2000) and human (Peduto Eberl et al, 2000 in press) colon cancer cell lines, blockade of caspase activity by zVAD-fmk had no effect on cells treated with bosentan in the absence of FasL, whereas apoptosis was completely inhibited by zVAD-fmk in the presence of both bosentan and FasL, involving PKC blockade but not the ceramide pathway. In human colon carcinoma cells, bosentan treatment did not modify either short or long forms of FLIP (Peduto Eberl et al, 2000 in press).

In conclusion, the different components of the endothelin system were examined in human glioblastoma. Expression of ECE-1 and PPET-1 in tumor vasculature provides a local source of ET-1 involved in a paracrine role in tumor cells via ET_B receptors and an autocrine function in tumor-associated vasculature via ET_A receptors. In human glioblastoma cell lines, the ET-1 system was not involved in tumor prolifera-

tion but ET-1 was a survival factor, possibly acting through ERK phosphorylation and the PKC pathway, resulting in a stabilization of the short form of FLIP protein.

Materials and Methods

Human Tissues

Human glioblastoma tissue was randomly selected from surgical diagnostic biopsies and subtotal resection specimens, and either fixed in 4% buffered paraformaldehyde and embedded in paraffin, or frozen in liquid nitrogen and stored at -80°C . Paraffin-embedded ($n = 8$) and unfixed frozen ($n = 4$) samples were used for ISH and immunohistochemistry and for ET-1 binding, respectively.

Immunohistochemistry

Paraffin-embedded sections (5 μm thick) of human glioblastoma were deparaffinized in xylene and isopropanol and endogenous peroxidase was inactivated in 3% hydrogen peroxide in methanol. Sections were washed in PBS, incubated with primary antibodies: human anti-CD31 and anti-CD68 (Dako, Zug, Switzerland), anti- α -SMA (Sigma, Buchs, Switzerland), monoclonal anti-ET-1 (ABR; Affinity Bioreagents, Alexis Corporation, Läufeligen, Switzerland), polyclonal anti-ET-1 (Peninsula, Brunschwig, Basel, Switzerland), anti-ECE-1 (antiserum 473-17-A; (Korth et al, 1999), or to *Ulex Europaeus* (Vector, Alexis Corporation) and subsequently exposed either to avidin-biotin complex (ABC; Dako) or to peroxidase-conjugated secondary immunoglobulins, according to the manufacturers' instructions. Peroxidase activity was visualized using 0.035% diaminobenzidine (Fluka, Buchs, Switzerland) as a chromogen and slides were counterstained with hematoxylin. Reactions performed without the primary antibody were used as controls for nonspecific reactions (not shown).

ISH and [^{125}I]-ET-1 Binding

ISH for the paraffin sections was performed as previously described (Egidy et al, 2000), with antisense and sense probes for PPET-1, ECE-1, and ET_A and ET_B receptors. Sense probes yielded no specific labeling in any tissue (not shown). *Ulex Europaeus* and CD31 labeling were performed on some sections immediately after ISH, before immersion into the Kodak NTB2 photographic emulsion. Slides were exposed for 4 weeks (ECE-1 and ET_A and ET_B receptor probes) or 8 weeks (PPET-1 probe). Frozen glioblastoma sections (7 μm thick) were used to evaluate binding of [^{125}I]-ET-1 as previously described (Egidy et al, 2000). Briefly, consecutive sections fixed in 4% formaldehyde in PBS were exposed to 100 pM [^{125}I]-ET-1 (2125 Ci/mmol) in 50 mM Tris-HCl buffer, pH 7.5, containing 120 mM NaCl, 5 mM MgCl₂, 40 mg/l of bacitracin, 1 mM phosphoramidon, and 1% BSA (fraction V, protease-free). Nonspecific binding was determined similarly in consecutive sections incubated in the presence of 1

μM unlabeled ET-1 (Bachem, Bubendorf, Switzerland). To characterize the receptor subtypes, sections were incubated in the presence of 1 μM BQ123, a selective ET_A receptor antagonist, or 0.2 μM sarafotoxin 6c, a selective ET_B receptor agonist.

Cell Culture and Treatments

The human glioblastoma cell lines LN18, LN215, LN2308, and LN319 were cultured in DMEM with 4.5 g/l of glucose, supplemented with 10% FCS. Cells were grown as monolayers to 50% confluence, washed, and incubated at 37°C with the endothelin-receptor antagonist, bosentan (kindly supplied by Dr. M. Clozel, Actelion, Basel, Switzerland), in the presence or absence of Neuro2A FasL-containing supernatant (Rensing-Ehl et al, 1995) (2 volumes of culture medium:10% FCS and 1 volume of FasL-containing supernatant) for 24 hours or 48 hours. Rat glioblastoma Fas-positive F98 and Fas-negative F98ZH cells (Ambar et al, 1999) were used as controls. Cell culture reagents were purchased from Gibco-BRL (Life Technologies, Basel, Switzerland) and bisindolylmaleimide IX from Calbiochem (Juro, Luzern, Switzerland).

Thymidine Incorporation

Cells were grown to 75% confluence. FCS was then removed from the culture for 24 hours. Increasing concentrations of ET-1 (Bachem) were added together with 0.2 $\mu\text{Ci}/\text{well}$ of [^3H]-thymidine (Amersham Pharmacia, Dübendorf, Switzerland) for 3, 5, or 48 hours, and incorporation was quantitated as previously described (Juillerat-Jeanneret et al, 1992).

Determination of p44/42 MAP Kinase/ERK Phosphorylation

Cells were grown in DMEM containing 10% FCS, deprived of serum for 24 hours, and exposed either to ET-1 (Bachem) for 5 to 120 minutes or to 10% FCS for 120 minutes. Cell cultures were extracted using 0.1% Triton X-100 in the presence of protease inhibitors (Roche Boehringer, Rotkreuz, Switzerland) and the extracts were submitted to electrophoresis. After transfer, the membrane was probed using a phospho-p44/42 MAP kinase monoclonal antibody (clone E10; New England BioLabs, Bioconcepts, Allschwil, Switzerland).

Cell Death Evaluation

Apoptosis was detected using the Cell Death Detection ELISA^{PLUS} (Roche Boehringer), a photometric enzyme-linked immunoassay for quantitative in vitro determination of cytoplasmic histone-associated DNA-fragments, as previously described (Peduto Eberl et al, 2000).

Measurement of Endothelin Secretion

The quantitative determination of ET-1 in cell culture supernatants at 24 hours or 48 hours was performed

Table 2. Specific Primers for the Amplification of ECE-1, PPET-1, PPET-3, ET_A, ET_B, LongFLIP and ShortFLIP cDNA in Glioblastoma Cells

Gene	Primer sequences for RT-PCR	Size of amplified fragments
ECE-1	Sense: 5'AGTATGACAAGGACGGGAACC 3' Antisense: 5'CTTACCAGACTTCGCACTTGTG 3'	459 bp
PPET-1	Sense: 5'TTGAGATCTGAGGAACCCG 3' Antisense: 5'TACGGAACAACGTGCTCG 3'	354 bp
PPET-3	Sense: 5'GGGCAGGAGCAGGCGG 3' Antisense: 5'AGGTAGATGGAGCGAGGG 3'	445 bp
ET _A	Sense: 5'GATCCTGTCCTTTATCCTGGC 3' Antisense: 5'CATTTCATGGGGACCGAGG 3'	576 bp
ET _B	Sense: 5'TACAGAAAGCCTCCGTGGG 3' Antisense: 5'GGGGAAGCCAGCAGAGGG 3'	477 bp
ECE-1 Isoforms	Common antisense: 5'GCTGAAGAAGTCA-TGGCAGGGGTC 3'	
ECE-1a	Sense: 5'CAGCCCTGATGCCTCTCCAG 3'	353 bp
ECE-1b	Sense: 5'CCCTGCTGTGCGCGCTGGGG 3'	347 bp
ECE-1c	Sense: 5'CGGAGCACGCGAGCTATGATG 3'	348 bp
ECE-1d	Sense: 5'ATGGAGGCGCTGAGGGAGTCC 3'	369 bp
IFLIP	Sense: 5'GGGAAGCTTATGTCTGCTGAAGTCATC 3' Antisense: 5'GGGGAATTCCTTCTGATTCCTGAATGG 3'	391 bp
sFLIP	Sense: 5'CCAGAAGGAAGAGCCCATATA 3' Antisense: 5'CCTCACCATTCTCTGCCATCA 3'	507 bp
GAPDH	Sense: 5'TGACCCCTTCATTGACCTCAACTAC 3' Antisense: 5'AAAGTTGTCATGGATGACCTTGG 3'	403 bp

ECE-1, endothelin-converting enzyme-1; FLIP, FLICE/caspase-8 inhibitory protein; IFLIP, long FLIP; sFLIP, short FLIP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

using a chemiluminescent enzyme-linked immunoassay (QuantiGlo; R&D Systems, Wiesbaden, Germany), according to the supplier's instructions. Cross-reactivity with human big-ET-1, ET-2, and ET-3 was less than 0.1%, 27.4%, and 7.8%, respectively, according to the manufacturer's specifications.

mRNA Analysis by RT-PCR

Total RNA was isolated from cell cultures using the Trizol reagent (Gibco-BRL). RT-PCR was performed according to standard procedures. Sequences of the primers used for each cDNA are given in Table 2. As controls, amplification reactions were performed with pairs of primers specific for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control reactions for RT-PCR analyses were carried out from non-reverse-transcribed RNA samples. No amplification was observed for any of the RNA samples tested (not shown). Twenty-eight cycles were carried out and amplified products were analyzed on 2% agarose gel.

Determination of Long and Short Forms of FLIP and Caspase-8 by Western Blotting

Confluent cell cultures were extracted using 0.1% Triton X-100 and protease inhibitors, and cell extracts were submitted to electrophoresis under nonreducing conditions on a 12% polyacrylamide gel. After transfer, the membrane was probed using the human FLIP AL148 polyclonal antibody, which was raised against a peptide spanning the amino acid sequences 2 to 26,

which are common to both the short and long forms of FLIP (Irmier et al, 1997). Caspase-8 determination was performed as previously described (Irmier et al, 1997).

Statistical Analysis

Means and standard deviation (SD) were calculated. Statistical significance was assessed using a two-tailed Student's *t* test. Experiments were repeated at least three times with comparable information, unless otherwise indicated.

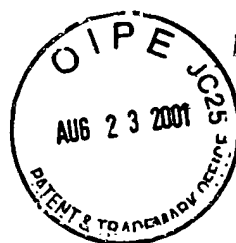
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Histopathology



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Augmented expression of endothelin-1, endothelin-3 and the endothelin-B receptor in breast carcinoma

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Aims: Endothelins (ETs) are peptides expressed in many tumours which may stimulate angiogenesis and desmoplasia. Because ETs have not been extensively studied in mammary neoplasia, we assessed ET protein and mRNA expression and receptor mRNA expression in normal and neoplastic breast tissues.

Methods and results: Tissues from five normal breasts, six fibroadenomas, seven ductal carcinomas in situ (DCIS) and 25 invasive carcinomas were stained with anti-ET-1 and anti-ET-3 antibodies and analysed using a grading system. ET-1, ET-3, ET_A and ET_B mRNA expression was assessed by quantitative RT-PCR from eight carcinomas and five normals. Weak staining for ET-1 and ET-3 was

detected in all normals. Moderate to strong staining was seen in 72% and 64% of carcinomas for ET-1 and ET-3, respectively. Most fibroadenomas showed weak positivity for ET-1 (83%) and ET-3 (67%). ET-1 and ET-3 mRNA levels were upregulated in carcinomas compared with normal breast. No ET_A mRNA was not detected in any tissue. ET_B mRNA was detected in normal breast and was increased in carcinomas.

Conclusion: These results suggest that the ET system is altered in breast carcinomas and this may be of importance in the progression from in-situ to invasive carcinoma.

Keywords: breast, carcinoma, endothelin-1, endothelin-3, endothelin-B receptor

Introduction

In 1988, Yanagisawa and colleagues isolated an extremely potent vasoconstricting substance from porcine aortic endothelium which is now referred to as endothelin.¹ Endothelins (ETs) are a family of four short peptides, each of approximately 21 amino acids and are commonly referred to as ET-1, ET-2, ET-3 and ET-4. ET-1 may be critical in the pathophysiology of essential hypertension.² However, ET-1 is now known to have widespread tissue distribution and diverse physiological roles. For example, ET-1 promotes

steroidogenesis in Leydig cells³ and has neurotransmitter and neuromodulator activity in the retina.^{4,5} Also, ET-1 stimulates melanogenesis *in vivo* and *in vitro*.⁶ In addition, ETs appear to have critical roles in embryogenesis. *ET-1* gene knockout mice die very shortly after birth due to respiratory failure and have severe midline craniofacial abnormalities.⁷ ET-2 is produced predominantly in the kidneys and intestine and appears to have limited physiological function(s). ET-3 is produced by a variety of cell types and organs including kidneys, brain, and retina and is involved in the proliferation and development of several cell types. Increased ET-1 mediated expression of several oncogenes, such as *c-fos* and *c-jun*, has been demonstrated.⁸⁻¹⁰

At least three ET receptors exist and are now referred to as ET_A, ET_B and ET_C.¹¹ In the breast, ET receptors are located on stromal fibroblasts, not epithelial cells.¹² ET_A shows selectivity for ET-1 whereas ET_B binds both ET-1

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and ET-3. In both instances, the receptor–ligand interaction stimulates production of inositol 1,4,5-trisphosphate and increased intracellular calcium.¹³ There is also evidence that ETs operate through the cAMP/G protein second messenger system.¹¹

Alteration of ETs and/or their receptors may be of importance in the progression of several neoplasms. ETs directly stimulate DNA synthesis and are mitogenic to several cell types including fibroblasts, melanocytes, vascular smooth muscle and endothelium *in vitro*.^{9,11,13–18} Also, it has been shown that immunohistochemically detectable ET-1 expression in human gliomas correlates with the degree of tumour vascularity.¹⁹ Pedram *et al.*¹⁸ have shown that both ET-1 and ET-3 act via ET_A and ET_B receptors and signal through protein kinase C to induce synthesis of vascular endothelial growth factor (VEGF), a key angiogenic factor secreted by vascular smooth muscle cells. In recent years, increased ET-1 mRNA expression has been documented in many tumours, including carcinomas of breast,²⁰ prostate,²¹ liver,²² and some mesenchymal tumours, including meningiomas²³ and phyllodes tumours of the breast.²⁴

ET-1 is normally synthesized in small amounts by breast epithelial cells. It is not known if ET-3 is physiologically synthesized in the breast. ET-1 but not ET-3 immunoreactivity has been documented in breast cancer.^{11,25} ET-1 immunoreactivity does not appear to be an independent prognostic factor in breast carcinoma.²⁶ Very little is known about ET-3 expression in human neoplasia and to the best of our knowledge, endothelin receptor expression has not specifically been studied in breast cancer. However, increased and decreased ET receptor expression has been documented in other neoplasms. The changes seen in various neoplasms are, however, nonuniform. For example, augmented ET-1 mRNA expression in endometrial carcinomas is associated with decreased ET_A mRNA expression, suggesting a counter-regulatory effect.²⁷ Nelson *et al.* have shown increased ET-1 production in association with decreased ET_B receptor mRNA expression in advanced prostate cancer.²¹ These results suggest that mediation of ET action can be tissue and/or tumour specific.

In order to assess the possible role(s) of endothelins in the pathogenesis of breast neoplasms, we investigated immunoreactive ET-1 and ET-3 distribution as well as mRNAs for ET-1, ET-3, ET_A and ET_B by semiquantitative RT-PCR.

Materials and methods

Formalin-fixed, paraffin-embedded tissues were studied from 25 consecutive breast carcinomas (invasive duct

carcinomas of no special type), six fibroadenomas and two high-grade ductal carcinoma in situ (DCIS) received at the London Health Sciences Centre. Adjacent normal breast tissue and five breast tissue samples from reduction mammoplasty were used as controls. Areas with DCIS were further identified and studied in tissues adjacent to five invasive carcinomas. Frozen tissue was available for mRNA studies from 13 cases (eight carcinomas, five reduction mammoplasty specimens).

HISTOLOGICAL ANALYSIS AND IMMUNOHISTOCHEMISTRY

All specimens were originally assessed on haematoxylin and eosin stained slides by a pathologist. Adjacent sections (5 µm) were picked up on positively charged slides for immunohistochemistry. Immunohistochemical analysis for ET-1 and ET-3 was performed with polyclonal antibodies (Penninsula Laboratories, Belmont, CA) using the ABCTM method and haematoxylin counterstains. Specificity of the antibodies was established by reabsorbing with specific peptides. For routine staining in the negative controls nonimmune rabbit serum was used. The immunohistochemical staining process has been previously described.²⁸ All slides were graded using a scoring system: grade III, 75–100% cytoplasmic positivity of the tumour cells; grade II, 25–75% cytoplasmic positivity of tumour cells; and grade I, <25% cytoplasmic positivity of the tumour cells.

SEMIQUANTITATIVE RT-PCR

RNA isolation

TRIZOLTM reagent was used to isolate RNA from the breast tissue. Briefly, following homogenization, RNA was extracted with chloroform followed by centrifugation to separate the solution into aqueous and organic phases. RNA was recovered from the aqueous phase by precipitation with isopropyl alcohol and suspended in DEPC-water. Quantification of RNA was performed by determining the absorbance at 260 nm and 280 nm.

First strand cDNA synthesis

First strand cDNA synthesis was performed using Superscript-IITM system reverse transcription was carried out by the addition of MMLV-reverse transcriptase and dNTPs at 42 °C for 50 min in a total reaction volume of 20 µl.

Polymerase chain reaction (PCR)

The amplification was carried out using the following cDNA sequences. For ET-1, primer 1 (antisense, 5'-AAGTCCCAGCCAGCATGGAGAG-CG-3' and primer 2

(sense, 5'-CGTTGCTCCTGCTCCTTCCTTGATGG-3') with a predicted product size of 543 bp and for ET-3, primer 1 (antisense, 5'-GCTGGTGGACTTTATC-TGTCC-3') and primer 2 (sense 5'-TTCTCGGGCTCACAGTGACC-3') with a predicted product size of 477 bp were used.²⁹⁻³¹ For ET_A primer 1 (antisense, 5'-TTCGTCATGG-TACCCTTCGA-3') and primer 2 (sense 5'-GATACTCG-TTCCATTCAT-GG-3') with a predicted product size of 546 bp and for ET_B primer 1 (antisense 5'-TTCACCT-CAGCAGGATTCTG-3') and primer 2 (5'-AGGTGTG-GAAAGTTAGAACG-3') with a predicted product size of 475 bp were used.³²⁻³⁴ Reactions were performed in 30-µl volumes containing 1.5 mM MgCl₂. The amplification for ET-1 and ET-3 were carried out as follows: 1 min at 94 °C (denaturation), 1 min at 60 °C (annealing) and 3 min at 72 °C (extension) for 40 cycles. It has been previously shown that in this reaction the PCR amplification is log-linear in this range.²⁹⁻³¹

For the receptor genes 54 °C was used as the annealing temperature and 35 cycles of amplification were performed. Simultaneously, a control gene (GAPDH) was amplified in a separate set of tubes using the same RT-product (primer set: 5'-CAACTT-GATCCACGTTCCACC-3' and 5'-GAAGAGCCAAGGA-CAGGTAC-3') using similar cycling parameters with a predicted product size of 270 bp. The amplification products were analysed on a 3% agarose gel in 1 × TAE buffer.

Southern hybridization

The specificity of the amplifications was confirmed by southern blot following transfer of the PCR product from the gel onto nylon membranes after denaturation and neutralization. Hybridizations were performed with biotinylated amplification product specific (ET-1: 5'-CAAAGACCACAGACCAAGGG-3', ET-3: 5'-CCTGCA-CAGCCTGGAAATGC-3' ET_A: 5'-CGAGGTCATGAGGC-TTTTGG-3' and ET_B: 5'-TGCAGACCTTCCGCAAG-CACG-3') oligoprobes.^{29-31,33,34} A similar amplification product specific oligoprobe (5'-GAGGGCAGGAGC-CAGGGCTGGGCA-3') was used for GAPDH. The detection was carried out using a NBT/BCIP system.

Quantification

Quantification was performed by serial dilution slot-blot hybridization and densitometry of the products from the upstream of amplification onto nylon membranes (PhotogeneTM, Canadian Life Technologies Inc., Burlington, Ontario, Canada). Hybridization was carried out using the biotinylated oligoprobes as described above and the detection was carried out using a NBT/BCIP system. The plates were analysed by a Hewlett-Packard 4C scanner and using MochaTM software

(Jandel Scientific, San Rafael, CA). The densitometric values were expressed as ratios to the control gene.

Results

IMMUNOHISTOCHEMICAL ANALYSIS

In all of the normal breast tissues (in reduction mammoplasties and in normal breast tissue flanking tumours), weak (grade I) diffuse staining for both ET-1 and ET-3 was present in the duct and acinar epithelial cells. Endothelium of larger blood vessels stained positively for both peptides. Smooth muscle cells of the larger blood vessels were positive for ET-1 and ET-3. No staining of myoepithelial cells was detected. One of six (17%) fibroadenomas demonstrated grade II staining for ET-1 where as the remaining five (83%) showed grade I staining for ET-1. Similarly, two fibroadenomas (33%) were moderately positive (grade II) for ET-3 whereas the remaining four fibroadenomas (67%) showed grade I staining for ET-3. Nine carcinomas (36%) showed grade III staining of the tumour cells, nine carcinomas (36%) showed grade II staining and the remaining seven (28%) carcinomas showed grade I staining. The staining intensity of ET-1 and ET-3 correlated with each other, i.e. cases which were strongly positive for ET-1 stained similarly with ET-3. Nine carcinomas (36%) showed grade III staining of the tumour cells, seven (28%) carcinomas showed grade II staining and nine carcinomas (36%) showed grade I staining. All DCIS was high grade, comedo-type. Two (29%), three (42%) and two (29%) cases of DCIS exhibited grade I, grade II and grade III staining, respectively, for ET-1. Three (42%) and four (58%) cases of DCIS showed grade II and grade III staining for ET-3, respectively (Figure 1).

No tumour was completely negative for either ET-1 or ET-3. All tumour tissues showed staining intensity in the blood vessels (endothelium and vascular smooth muscle) similar to that of normal breast tissue. The negative controls were uniformly negative. Statistical analysis by chi-square test showed significantly ($P < 0.005$) higher immunoreactivity in the carcinomas and DCIS for both peptides compared to the normals tissue. The expression of immunoreactive proteins in fibroadenomas were, however, not different from normal breast tissues. The expression of ET-1 and ET-3 were further higher in invasive carcinomas compared to DCIS cases areas was significant ($P < 0.02$).

Significantly higher ET-1 and ET-3 mRNAs were present in the malignant breast tumours as compared to the normal breast tissue. No evidence of ET_A mRNA was detected either in normal or in the neoplastic breast

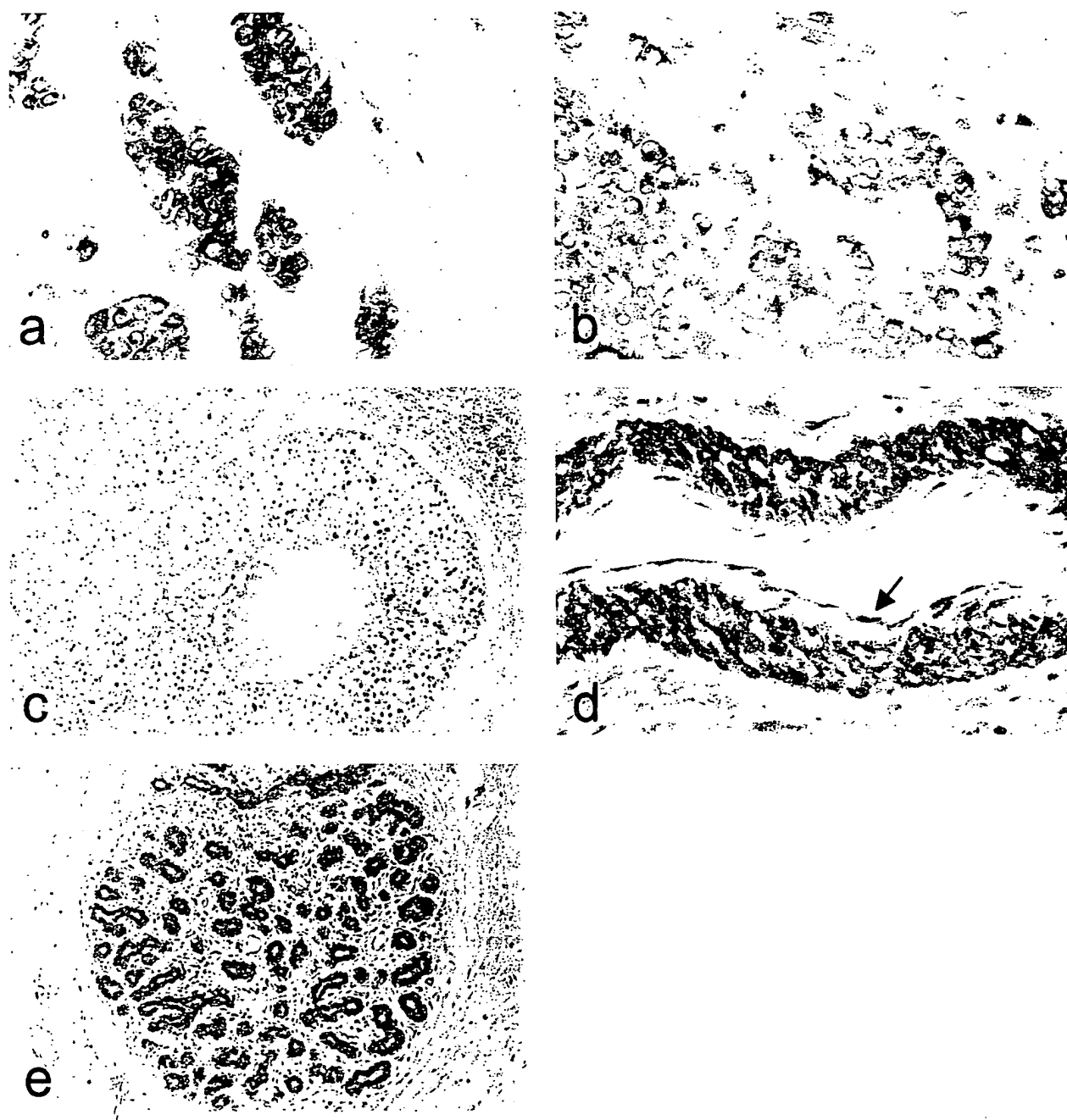


Figure 1. Infiltrating duct carcinoma with positive immunohistochemical staining for ET-1 (a) and ET-3 (b). c, ductal carcinoma in situ with ET-1 immunoreactivity. Vascular smooth muscle and endothelium (arrow) with positive immunoreactivity for ET-1 (d). Weak positive staining for ET-1 in normal breast (e). Haematoxylin counterstain.

tissue. ET_B mRNA was present in the normal breast tissue and was augmented in breast carcinomas (Figure 2).

No correlation of the immunohistochemical stain or mRNA expression was demonstrated with tumour grade, stage, size and lymph node status or hormone receptor status.

Discussion

We have shown the presence of immunohistochemically detectable ET-1 and ET-3 in normal breast epithelium. In addition, cytoplasmic ET-3 was identified in endothelium and vascular smooth muscle. We have demonstrated significantly increased ET-1 and ET-3

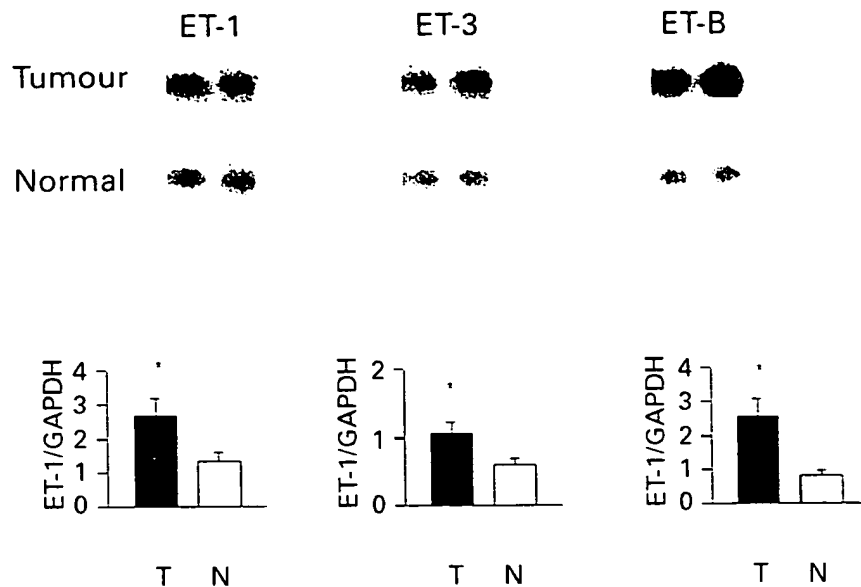


Figure 2. a. Representative Southern blots of PCR products following amplifications from breast carcinomas (tumour) or normal breast tissues (normal). b. quantitative RT-PCR data expressed as a ratio to the control gene GAPDH. N, normal breast tissue; T, breast carcinomas ($n = 8$). * $P < 0.05$.

immunoreactivity and mRNA expression in tumour cells compared to the non-neoplastic normal tissue. There is significantly greater ET-1 and ET-3 expression in carcinomas as compared with fibroadenomas at both the messenger RNA and protein levels. This study has further demonstrated that, in the breast, ET_B receptor mRNA is augmented in breast carcinomas as compared to the normal breast tissue. ET_A receptor mRNA was not detected in the normal or neoplastic breast tissues. We did not demonstrate an association between ET expression and classic prognostic factors such as patient age, tumour size, lymph node status, or hormone receptor expression. Yamashita *et al.* have previously demonstrated increased ET-1 protein has been demonstrated from the tissue extracts of breast carcinoma.²⁰ In that study, there was no correlation with ET-1 expression and patient age, tumour size, lymph node status, histological tumour type or hormone receptor expression. Increased immunoreactive ET-1 has been previously described in breast carcinoma and was found to predict for metastasis.²⁵ This is the first study investigating ET-3 or ET-receptor expression in mammary neoplasms.

The exact physiological role of ET peptides in the duct epithelium of normal breast tissue remains unclear. Because ETs function as autocrine factors with potent mitogenic activity, they may participate in development of the breast,¹¹ as has been suggested in the other organs. For example, they are important for distal migration of enteric neurones and melanocytes.³⁵ Several physiological or pathological stimuli may increase production of ETs. Of relevance to the current study, factors which may stimulate ET production and

release from breast cancer cell lines include TGF- α ,¹² IL-6,³⁶ cortisol, bombesin³⁷ and prolactin.¹²

There is no evidence to suggest a direct role of endothelins in carcinogenesis although there is abundant evidence that ET-1 directly stimulates DNA synthesis.¹³⁻¹⁶ ETs have been shown to be mitogenic to fibroblasts,^{9,11} melanocytes,¹⁷ vascular smooth muscle and endothelium *in vitro*.^{13,16,18} Activation of endothelin receptor-coupled pathways in vascular smooth muscle cells and Swiss 3T3 cells has also been shown to enhance expression of the protooncogenes *c-fos* and *c-myc*.⁸⁻¹⁰

It is of interest to note that the pure DCIS specimens (and DCIS flanking invasive tumours) showed staining intensities intermediate between carcinomas and normal tissues when stained with anti-ET-1 and anti-ET-3 antibodies. Although the possibility of a small sample size cannot be totally excluded, this finding raises an intriguing possibility that upregulation of ET genes may be associated with tumour progression and development of invasive phenotypes. It is known that ET-1 induces release of interleukin-6 (IL-6) from fibroblasts and/or endothelial cells.¹¹ IL-6, in turn, may contribute to the development of invasive phenotypes.^{38,39} Furthermore, ET-1 has been shown to induce expression of tissue-type plasminogen activator and possibly type IV collagenase,⁴⁰ enzymes which are important for dissolution of basement membranes promoting invasion.

A recent study suggests that endothelins and their receptors participate in tumour-associated angiogenesis. ET-1 has been shown to directly stimulate endothelial cell proliferation *in vitro*.^{13,16} Stiles *et al.*¹⁸

studied immunohistochemical expression of ET-1 in human gliomas and found that expression of both ET-1 and ET-3 act via ET_A and ET_B receptors and signal through protein kinase C to induce synthesis of VEGF by vascular smooth muscle cells. VEGF is a well established and very important mediator of angiogenesis. Furthermore, antagonism of ET_A and ET_B receptors with specific inhibitors (BQ-123 and IRL-1038, respectively) prevented VEGF synthesis. By virtue of their stimulatory effects on angiogenesis ETs may have additional effects on tumour progression and invasion.

Interestingly, tumours with dense desmoplastic reactions exhibited increased ET-1 and ET-3 expression, both by immunohistochemistry and RT-PCR (data not shown). It is possible that this fibrous reaction is, at least in part, mediated by ETs. ET-1 has been shown to have a mitogenic effect on breast fibroblasts.⁴¹ It is known that ET_A receptors are expressed on breast fibroblasts which are likely stimulated by epithelial-derived ET-1. This epithelial/stromal interaction does not appear to be unique to breast carcinomas. For example, in osteoblastic metastases of prostatic adenocarcinoma, there is increased ET-1 production but decreased ET_B receptor expression on tumour cells.²¹ ET-1 stimulates production of alkaline phosphatase and appears to mediate, at least in part, the osteoblastic response to metastases.⁴² It has further been suggested that ET-1, through ET_A, is thought to be important in mediating the desmoplastic response in colonic adenocarcinoma.⁴³

Only one of six fibroadenomas showed moderate staining for ET-1 and two of the six for ET-3, while the remainder showed weak to no staining. This is in keeping with an earlier study by Yamashita *et al.*²⁴ It is interesting to note that mammary phyllodes tumours show significant ET-1 production by the epithelial component.²⁴ Whether locally aggressive nature of phyllodes tumours as compared with fibroadenomas is related to ET-1-induced proliferation needs to be confirmed by further studies. This is the first published evidence which we are aware of which demonstrates increased ET-3 production and ET_B expression in breast cancer. Although some differences exist, for the most part, these two peptides have similar biological actions. The presence of ET_B receptors in normal breast tissue and its augmentation in malignancy implies possible importance of this receptor in breast tissue. This is in contrast to the findings in advanced prostate carcinoma where ET_B mRNA expression is reduced in association with increased ET-1 mRNA expression.²¹ In localized melanoma, there is significant expression of ET_B mRNA, which decreases in metastatic melanoma.⁴⁴ Thus, it appears that ET receptor expression varies with respect to tumour type and possibly tumour stage. It is tempting

to speculate that expression of ETs in breast carcinomas may have potential utility as a prognostic marker. Such speculation, however, has to be confirmed by larger series.

In summary, this study has demonstrated significantly increased expression of peptide hormones ET-1 and ET-3 and one of their receptors, ET_B in the breast carcinomas. Accumulating evidence suggests that alteration of endothelin system may be of importance in the pathogenesis of breast carcinoma although more studies are required to elucidate the precise role(s) of the endothelin system in breast carcinoma.

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